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Enhanced Bleomycin-Induced DNA Damage and Cytotoxicity with Calmodulin Antagonists

JOHN S. LAZO, WILLIAM N. HAIT, KATHERINE A. KENNEDY, I. DEBORAH BRAUN, AND BORISLAV MEANDŽIJA

The Departments of Pharmacology (J.S.L., I.D.B., B.M.) and Medical Oncology (W.N.H.), Yale University School of Medicine, New Haven, Connecticut 06510 and the Department of Pharmacology, The George Washington University, Washington, D. C. 20037 (K.A.K.)

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SUMMARY

A wide variety of structurally different calmodulin antagonists enhanced the cytotoxicity of bleomycin A_2 to leukemic L1210 cells. This potentiation occurred with nontoxic concentrations of calmodulin antagonists. The most potent blockers of L1210 calmodulin activity, melittin and mastoparan, were the most potent potentiators of bleomycin A_2 cytotoxicity. Less potent agents such as pimozide, a diphenylbutylpiperidine, trifluoperazine and chlorpromazine, phenothiazines, and W-7, a naphthalene sulfonamide, required higher concentrations for potentiation of bleomycin A_2-induced cytotoxicity, while homologs that lack anticalmodulin activity failed to increase the cytotoxicity seen with bleomycin A_2. The potentiation of bleomycin A_2 cytotoxicity was not due to an elevated cellular content of bleomycin A_2 or to inhibition of bleomycin A_2 inactivation. Using alkaline elution techniques, we found that pimozide increased bleomycin A_2-induced DNA damage in intact L1210 cells. Pimozide did not, however, directly increase the formation of reactive species by bleomycin as measured by single or double strand breakage of covalently closed circular DNA. Thus, the potentiation of bleomycin cytotoxicity by these agents appears to be mediated by an increased damage to cellular DNA; this may be due to inhibition of DNA repair. The hypothesized calmodulin-dependent mechanism was not shared by all agents that caused breaks in DNA because no potentiation in cytotoxicity was observed when calmodulin antagonists were combined with either etoposide or X-irradiation.

INTRODUCTION

Bleomycin, a complex mixture of glycoproteins, is an effective antitumor agent and is believed to act by causing single and double strand breaks in DNA (1). The single strand DNA breaks induced by bleomycin can be readily repaired (2, 3). In addition to DNA repair, it has been proposed that two other factors are important in determining tumor cell responsiveness to bleomycin: cellular uptake of the drug and inactivation by an aminopeptidase B-like enzyme called bleomycin hydrolase (1).

Recently, it has been suggested that agents altering intracellular calcium can increase the cytotoxic properties of bleomycin to tumor cells (4, 5), although the mechanism is unknown. A variety of calcium regulated events in eucaryotic cells appear to be mediated by the intracellular calcium-binding protein, CaM[1] (6–8). CaM may also have an important role in regulating cellular proliferation (8). Drugs that inhibit the function of CaM can arrest cellular proliferation and kill both normal and malignant cells (8–12). To determine if cellular processes regulated by CaM may affect bleomycin cytotoxicity, we investigated the potential cytotoxic interactions between CaM antagonists and bleomycin. Recently, Chafouleas *et al.* (13) presented data showing that a CaM antagonist can increase bleomycin cytotoxicity to Chinese hamster ovary cells and proposed that these agents may act by inhibiting repair of DNA. The effects of CaM antagonists on bleomycin accumulation or metabolism were not, however, investigated. We now report, from results with a malignant cell line, that a wide variety of structurally different CaM antagonists can potentiate the cytotoxicity of the principal component of the clinical mixture of bleomycins, BLM A_2.

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[1] The abbreviations used are: CaM, calmodulin, BLM A_2, bleomycin A_2; BLM dA_2, desamidobleomycin A_2; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; TEMED, *N,N,N',N'*-tetramethylethylenediamine; W-5, *N*-(6-aminohexyl)naphthalene sulfonamide; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulfonamide.

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MATERIALS AND METHODS

Cell-culturing techniques. L1210 murine leukemia cells were maintained in suspension culture with Fischer's medium and 10% horse serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were routinely assayed and found free of contamination by *Mycoplasma*. All studies were conducted with cells in exponential growth phase (2–3 × 10^5 cells/ml). The numbers of cells were determined with a Coulter model Z_{B1} counter (Hialeah, FL). Preparations of single cells (5 × 10^4 cells/2 ml) were exposed to 5 μM copper-free BLM A_2 in the presence or absence of CaM antagonists for 1 hr at 37°. The cells were then centrifuged at 200 × *g* for 5 min at 37°, the medium was removed, and the cell pellet was resuspended in 10 ml of Fischer's medium containing 15% horse serum. In some studies, cells were treated for 1 hr with 1 μM etoposide with or without a CaM antagonist or were X-irradiated (250 kV) in the presence or absence of a CaM antagonist and incubated for 1 hr.

Survival was determined by the method of Chu and Fischer (14) in which between 100 and 10,000 cells were resuspended in a culture tube with Fischer's medium, 15% horse serum, and 0.13% agar and incubated for 10 days at 37° in a 5% CO_2-95% air incubator. Colonies of approximately 250 μm or greater in diameter (approximately 8000 cells or 13 population doublings) were counted with a dissecting microscope (6.6×) after staining at 37° with 0.5 ml of a 1 mg/ml solution of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride for 24 hr (15). The average cloning efficiency of untreated control cells for all experiments was 94 ± 4%. The surviving fraction of cells was calculated from the ratio of the cloning efficiency of the experimental group to that of the untreated control. In this study, we have defined potentiation as the ability of a nontoxic concentration of a drug to increase the lethal effects of a second drug.

Drugs. Copper-free BLM A_2 was isolated by previously described methods (16) from Blenoxane (Bristol Laboratories, Syracuse, NY) and stored as stock solution (1 mM) in glass-distilled water at −20°. Purity (>95%) was monitored by previously described techniques (16). Clinical grade etoposide (Bristol Laboratories) was used. Melittin was obtained from Sigma Co. (St. Louis, MO) and mastoparan was purchased from Bachem, Inc. (Torrance, CA). Trifluoperazine, chlorpromazine, and chlorpromazine sulfoxide were gifts from Smith Kline Beckman (Philadelphia, PA); pimozide was kindly supplied by Dr. James Long (Janssen Pharmaceuticals, Piscataway, NJ). W-7 and W-5 were donated by Dr. John Hickman (University of Aston, Birmingham, England). High specific activity Cu^{2+} [*dimethylsulfonium*-^{3}H] BLM A_2 (43 Ci/mmol) was synthesized by New England Nuclear (Boston, MA) and stored in the copper form at 4° in 30% ethanol:H_2O. This radiolabeled material co-migrated with unlabeled BLM A_2 by high pressure and thin layer chromatography (16).

Preparation of L1210 cell CaM and analysis of CaM-dependent phosphodiesterase activity. CaM was prepared from L1210 cells by a modification of previously described methods (17). In this procedure, 1 × 10^9 L1210 cells were suspended in a phosphate-buffered solution (88 mM Na_2HPO_4, 23 mM NaH_2PO_4, 640 mM glycine, 1 mM EGTA, and 0.32 M sucrose, pH 6.0). The cells were sonicated at 50 W for 10 sec, incubated at 37° for 2 hr, placed in a boiling water bath for 5 min, and then centrifuged at 3600 × *g* for 20 min. The pH of the supernatant fraction was adjusted to 6.0, and recentrifugation was performed as above. The supernatant fraction was dialyzed for 12 hr against the sonicating buffer then concentrated to one-half its original volume by ultrafiltration through a CX-10 filter (Millipore Corp., Bedford, MA).

A 7.5% preparative polyacrylamide gel electrophoretic column (Shandon Southern Instruments, Inc., Sewickley, PA) was prepared in a phosphate buffer solution (pH 7.0) containing 40 mM NaH_2PO_4, 120 mM Na_2HPO_4, 640 mM glycine, 0.46 ml of TEMED, and 1 mM EGTA. An electrolyte buffer was prepared using 88 mM NaH_2PO_4, 23 mM Na_2HPO_4, 640 mM glycine, and 1 mM EGTA. Two ml of the partially purified CaM solution was applied to the gel column and the electrophoresis was begun at 25 mA for the first hour and 60 mA thereafter, using a constant current power supply (Bio-Rad Laboratories, model 500/200, Richmond, CA). Proteins were eluted with a solution of 0.1 M Tris buffer (pH 7.6) containing 1.0 mM $CaCl_2$ at a flow rate of 0.2 ml/min. One hundred 1-ml fractions were collected and each fraction was assayed for CaM activity. Using this method, a single peak of CaM was obtained eluting in fractions 35–50 and was found to be homogeneous by sodium lauryl sulfate-polyacrylamide gel electrophoresis.

The activity of CaM was assayed by its ability to activate a CaM-sensitive phosphodiesterase prepared from rat cerebrum as described by Levin and Weiss (18). The reaction contained 50 mM glycylglycine buffer (pH 8.0), myokinase (0.25 unit), pyruvate kinase (0.5 unit), 25 mM ammonium acetate, 3 mM $MgCl_2$, 400 μM cAMP, 100 μM $CaCl_2$, phosphodiesterase with or without 10 units of CaM, and various concentrations of drugs. Using our preparation of L1210 CaM, the activity of phosphodiesterase was increased a maximum of 5- to 10-fold. One unit of activity was defined as the amount of CaM required to achieve 50% of the maximum activation of phosphodiesterase. The effect of antagonists and their solvents on CaM-dependent activity of the enzyme was measured by the ability to inhibit the activation of phosphodiesterase in the presence of 10 units of CaM. At the highest concentrations of drugs studied, there was less than 5% inhibition of phosphodiesterase activity in the absence of CaM.

Measurements of cellular radioactivity. Exponentially growing L1210 cells were resuspended (7 × 10^6 cells/ml) in Fischer's medium with 10% horse serum and 10 mM HEPES (pH 7.4). Initial studies indicated that 10 mM HEPES did not alter the cytotoxicity of BLM A_2 to L1210 cells. [^{3}H]BLM A_2 was added to BLM A_2 to yield a final concentration of 1 μM [^{3}H]BLM A_2 (4.3 μCi/ml; specific activity, 4.3 μCi/nmol) which was added to cells in the presence or absence of 5 μM pimozide or 2 μM melittin. Cells were incubated with constant shaking at 37° in a water bath for various times. At the end of the incubation, 15 ml of an ice-cold phosphate-buffered 0.9% NaCl solution was added to each cell suspension and, after centrifugation at 1000 × *g* for 2 min, the supernatant fraction was removed and the cell pellet was resuspended in 350 μl of Fischer's medium with 10% horse serum. The cell concentration of this suspension was determined with a Coulter counter and triplicate samples of cells (100 μl each) were placed in a microcentrifuge tube containing 40 μl of 5% $HClO_4$ overlaid with 400 μl of a silicone oil/mineral oil (84:16, v/v) mixture. The tubes were centrifuged at 9000 × *g* for 15 sec and rapidly frozen in an ethanol/dry ice bath. The cellular pellet was separated from the free drug by cutting through the oil layer. The radioactivity in the bottom layer of the tube (cell fraction) was counted. Less than 1% of the total cellular radioactivity found in the cell pellet after washing with the NaCl solution and centrifugation was due to extracellularly associated radioactivity. The radioactivity in the cell pellet was determined using a liquid scintillation counter and corrected to disintegrations per minute using external standard techniques.

BLM hydrolase assay. BLM A_2 metabolism by BLM hydrolase was studied *in vitro* using a slight modification of previously described methods (16). Briefly, approximately 5 × 10^8 exponentially growing L1210 cells were centrifuged at 1000 × *g* for 3 min, washed twice with a phosphate-buffered 0.9% NaCl solution, and resuspended in 1 or 2 ml of a 0.1 M sodium phosphate solution (pH 7.2). The cells were then frozen, thawed, and sonicated three times for 5 sec. The homogenate was centrifuged at 20,800 × *g* for 45 min at 4° and the supernatant fraction was centrifuged at 105,000 × *g* for 1 hr at 4°. This solution was then assayed for protein by the method of Bradford (19) and stored at −70° until use. The metabolism of BLM A_2 *in vitro* was assayed by measuring the formation of BLM dA_2 as described previously (16). The rate of BLM dA_2 formation was linear for more than 1 hr.

Alkaline elution techniques. DNA strand breakage was determined by the methods of Kohn (20). L1210 cells were pretreated at 37° with [^{14}C]thymidine (0.01 μCi/ml, 1 μM) for 18 hr. After the 18-hr incubation, the medium was removed and new medium supplemented with 1 μM unlabeled thymidine was added. After a 6-hr incubation, the medium was removed and the cells were incubated for 1 hr in one of the following: drug-free medium, medium containing 50 μM BLM A_2, or

medium containing 50 μM BLM A_2 and 5 μM pimozide. The medium was removed and the cells were resuspended in ice-cold phosphate buffer and kept on ice in the dark until assayed. Internal standard cells were prelabeled with [^{3}H]thymidine (0.1 μCi/ml, 1 μM) in a similar manner and received 300 rads of X-irradiation at 4°. Experimental and internal standard cells were admixed and placed on a 2-μm filter (Nucleopore Corp., Pleasanton, CA) and eluted by the method of Kohn (20). The ^{3}H and ^{14}C in each sample were determined simultaneously using dual labeling techniques. Correction for quenching was made using external standards. Typical counting efficiencies for ^{3}H and ^{14}C under the conditions described were approximately 30 and 70%, respectively. The total amount of ^{14}C and ^{3}H in the sample was determined as the sum of the amounts in the individual fractions, the lysis fraction, the NaOH fraction, and the filter fraction. The fraction of ^{14}C retained (experimental cell DNA) was plotted against the fraction of ^{3}H retained on the filter (internal standard DNA).

Plasmid DNA breakage. The ability of pimozide to influence DNA breakage by BLM A_2 was determined using covalently closed circular DNA (21). Form I plasmid pAT 153 DNA was isolated (22) and stored as a stock solution at 4° in 40 mM Tris-acetate (pH 8.0) and 1 mM EDTA. Immediately prior to use, the stock DNA solution was diluted 4-fold with a 40 mM Tris-acetate solution (pH 8.0) to decrease the EDTA concentration. The incubation mixture (20 μl) contained 0.1 pmol of DNA, 25 mM dithiothreitol, 20 mM $MgCl_2$, 40 mM Tris-acetate (pH 8.0), and BLM A_2 with or without 10 μM pimozide. The molar ratio of BLM A_2 to pAT 153 DNA ranged from 0.3 to 100. The molecular mass of pAT 153 DNA used for this calculation was 2.3×10^6 daltons. The incubation was conducted for 20 min at 4° and stopped with the addition of 50 mM EDTA. The disappearance of form I DNA and the appearance of forms II and III DNA were assayed by gel electrophoresis with 1.2% agarose gels at room temperature for 4 hr at 4.5 V/cm. Following electrophoresis, the gels were stained with 1 μg/ml of ethidium bromide in 40 mM Tris-acetate for 30 min (21) and the DNA was visualized by UV irradiation.

RESULTS

Inhibition of CaM-dependent enzyme activity. We studied the ability of a variety of structurally diverse compounds to antagonize the stimulation of phosphodiesterase by CaM isolated from L1210 cells (Table 1). The two peptides, melittin and mastoparan, were the most potent antagonists of CaM activation of phosphodiesterase, having IC_{50} values below 1 μM. Pimozide was intermediate in inhibitory potency having an IC_{50} of 1.4 μM, while

TABLE 1

Inhibition of L1210-CaM-activated cyclic nucleotide phosphodiesterase

The activity of cyclic AMP phosphodiesterase was determined by the luciferin-luciferase technique of Levin and Weiss (18) in the presence or absence of 10 units of CaM. The highest concentration of each drug studied produced <5% inhibition of basal phosphodiesterase activity in the absence of CaM. Basal phosphodiesterase activity was 48 pmol of cyclic AMP hydrolyzed/ml/min and was increased 6-fold by CaM. IC_{50} is defined as the concentration required to inhibit the activation of CaM-dependent phosphodiesterase by 50%.

	IC_{50}
	μM
Melittin	0.12
Mastoparan	0.25
Pimozide	1.4
Trifluoperazine	10
Chlorpromazine	15
W-7	35
W-5	> 80
Chlorpromazine sulfoxide	>340

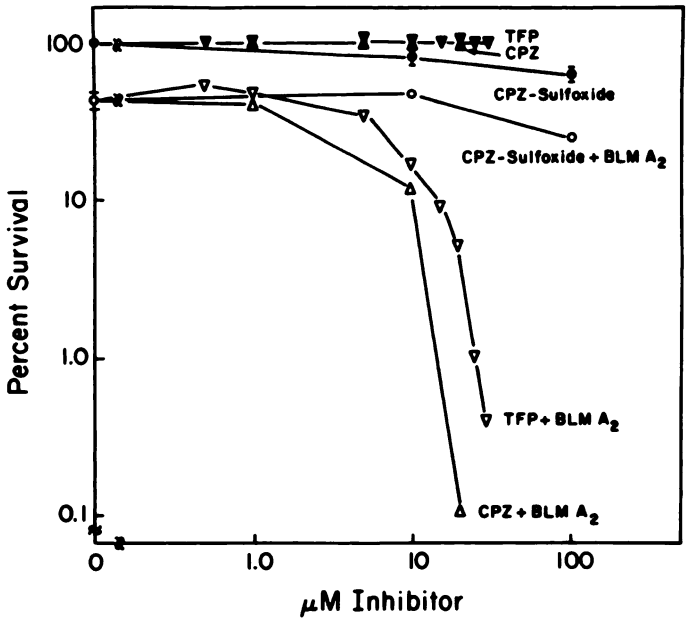

FIG. 1. *Potentiation of BLM A_2 cytotoxicity by phenothiazine CaM inhibitors*

Exponentially growing L1210 cells were exposed to various concentrations of trifluoperazine (*TFP*) (▼, ▽), chlorpromazine (*CPZ*) (▲, △), or chlorpromazine sulfoxide (●, ○) in the absence (closed symbols) or presence (open symbols) of 5 μM BLM A_2 for 1 hr. Cells were washed free of drugs, resuspended in drug-free medium, and placed in soft agar as described in Materials and Methods. The number of colonies was determined after incubation at 37° for 10 days. Each point represents the average of three to six determinations. There was less than 10% variability among all samples assayed at each point. Bars indicate standard error when greater than the size of the symbol.

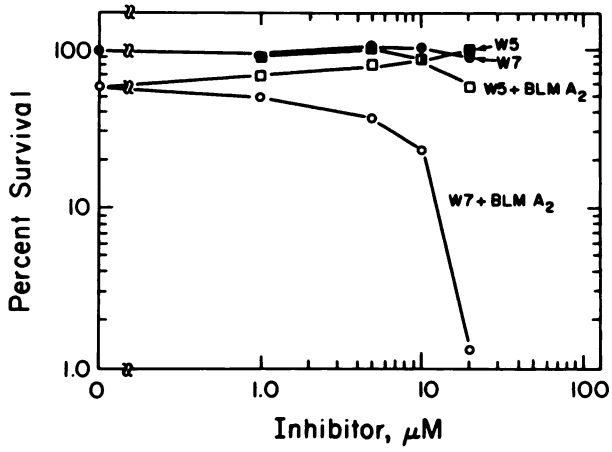

FIG. 2. *Potentiation of BLM A_2 cytotoxicity by naphthalenesulfonamide CaM inhibitors*

Exponentially growing L1210 cells were exposed to various concentrations of W-5 (■, □) or W-7 (●, ○) in the absence (closed symbols) or presence (open symbols) of 5 μM BLM A_2 for 1 hr. Cells were washed free of drugs, resuspended in drug-free medium, and placed in soft agar as described in Materials and Methods. The number of colonies was determined after incubation at 37° for 10 days. Each point represents the average of three determinations. There was less than 10% variability among all samples assayed at each point.

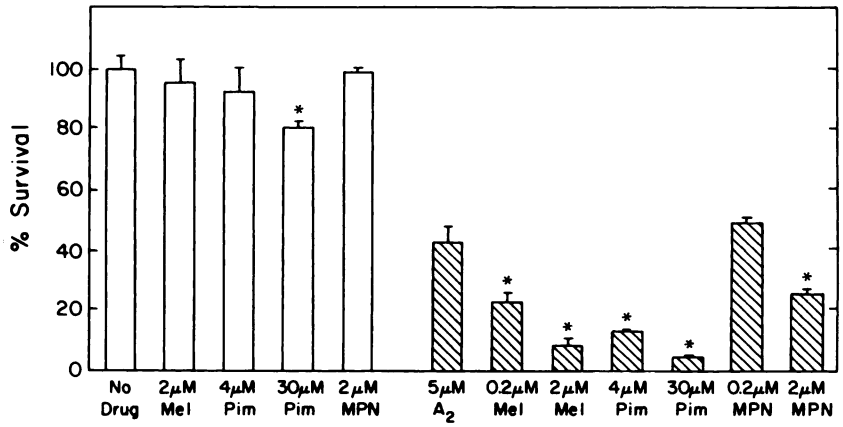

FIG. 3. *Potentiation of BLM A_2 cytotoxicity by diphenylbutylpiperidine and peptide CaM inhibitors*

Exponentially growing L1210 cells were exposed to CaM inhibitors alone (open bars) or in combination with 5 μM BLM A_2 (hatched bars) for 1 hr. The cell survival was assayed after cells were washed free of drugs by the colony assay described in Fig. 1. Each value is the average of three to nine determinations. Bars equal standard error. Treatment for 1 hr with 30 μM pimozide produced a significant decrease in survival compared to untreated cells as determined by a one-way analysis of variance and a least significant difference test ($p < 0.05$) and is indicated by the star. All other stars indicate a significant decrease in survival ($p < 0.05$) compared to cells treated with 5 μM BLM A_2 alone. *Mel*, melittin; *Pim*, pimozide; *MPN*, mastoparan.

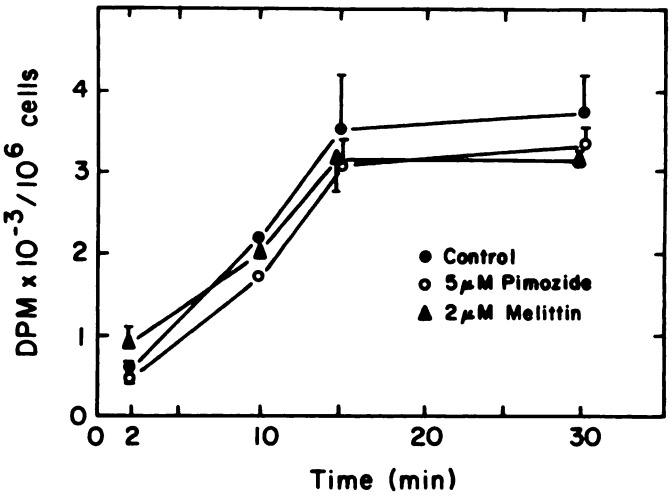

FIG. 4. *Effect of CaM inhibitors on [^{3}H]BLM A_2 cell association*

Exponentially growing L1210 cells were incubated at 37° with 1 μM [^{3}H]BLM A_2 in the absence (●) or presence of 5 μM pimozide (O) or 2 μM melittin (▲). Cell-associated radioactivity was assayed as described in Materials and Methods. Values at 10 min are the average of two determinations; all other points are the average of six determinations. Bars equal standard error.

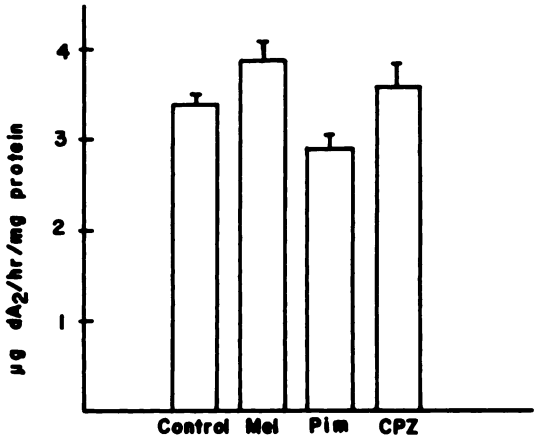

FIG. 5. *Effect of CaM inhibitors on BLM hydrolase activity*

In vitro BLM hydrolase activity was assayed as described in Materials and Methods. The concentrations of CaM inhibitors added to the postmicrosomal homogenate were 2 μM melittin (*Mel*), 5 μM pimozide (*Pim*), and 25 μM chlorpromazine (*CPZ*). Each point represents the average of three or four determinations. Bars equal standard error.

trifluoperazine, chlorpromazine, and W-7 were less potent, having IC_{50} values between 10 and 35 μM. W-5 and chlorpromazine sulfoxide were unable to inhibit by 50% the CaM activation of phosphodiesterase with the concentrations studied. These results are similar to previously reported data obtained with CaM from other tissues (7, 23).

Effects of CaM antagonists on BLM A_2 cytotoxicity. A 1-hr exposure of L1210 cells to concentrations of 30 μM or less of trifluoperazine or chlorpromazine or concentrations of 100 μM or less of chlorpromazine sulfoxide alone had no effect on cell survival (Fig. 1). When cells were incubated with 5 μM BLM A_2 alone for 1 hr and washed free of drug, a 50% decrease in cell survival was found. Addition of trifluoperazine or chlorpromazine to cells in the presence of 5 μM BLM A_2 caused a concentration-dependent decrease in cell survival; a 200–250-fold decrease in cell survival was seen with the addition of 20 μM chlorpromazine and 30 μM trifluoperazine, respectively. The use of higher concentrations of these CaM antagonists in combination with 5 μM BLM A_2 resulted in a greater enhancement of cell cytotoxicity, which exceeded the limits of detection of our assay. Simultaneous incubation of 5 μM BLM A_2 with 100 μM or less chlorpromazine sulfoxide, which was not a potent antagonist of CaM, produced no significant potentiation of BLM A_2 cytotoxicity.

Fig. 2 illustrates the results seen when L1210 cells were incubated for 1 hr with the naphthalene sulfonamide derivatives, W-7 and W-5. Both compounds were nontoxic to L1210 cells at concentrations of 20 μM or less. A significant potentiation of BLM A_2 cytotoxicity was observed when cells were exposed simultaneously for

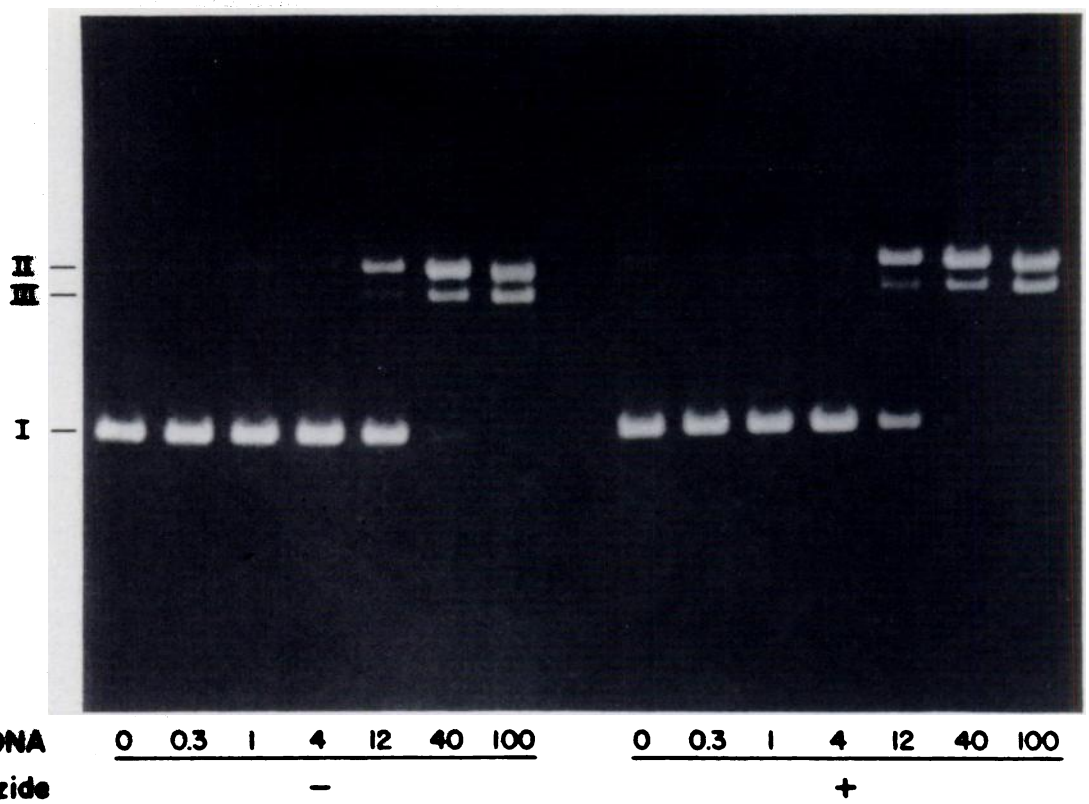

FIG. 6. *Effect of pimozide on DNA breakage by BLM A_2*

pAT 153 DNA (0.1 pmol) was incubated at 4° for 20 min with various concentrations of BLM A_2 in the absence or presence of 10 μM pimozide. The loss of covalently closed circular pAT 153 DNA (form I) and the appearance of single nicked (form II) and linear (form III) was determined by gel electrophoresis as described in Materials and Methods. This figure is representative of the results from three experiments.

1 hr to 5 μM BLM A_2 and W-7. Under identical conditions, potentiation was not detected when W-5 was added to cultured cells treated simultaneously with 5 μM BLM A_2.

Exposure of L1210 cells to 4 μM pimozide for 1 hr caused no reduction in cell survival, although a slight decrease was noted with a 30 μM concentration of this compound (Fig. 3). A 1-hr exposure to the peptides, melittin or mastoparan, at a concentration of 2 μM also had no effect on cell survival. A marked potentiation in BLM A_2 cytotoxicity was observed, however, when cells were co-incubated for 1 hr with 5 μM BLM A_2 in combination with 0.2 or 2 μM melittin, 4 or 30 μM pimozide, or 2 μM mastoparan.

CaM antagonists and [^{3}H]BLM A_2 cell association. To determine the mechanism of the enhancement of BLM A_2 cytotoxicity by CaM antagonists, we examined the effect of CaM antagonists on the rate and steady state levels of cell-associated BLM A_2 after incubation with [^{3}H]BLM A_2. No alterations in either the rate of cellular association or the steady state levels of radioactivity were observed with 5 μM pimozide or 2 μM melittin (Fig. 4). Thus, the potentiation by these agents could not be explained by an increased rate of cellular association or steady state levels of BLM A_2. The similar steady state levels of radioactivity and rate of cellular association also suggest that the rate of egress of BLM A_2 was unaltered.

Effect of CaM antagonists on BLM hydrolase activity. BLM A_2 can be inactivated by an aminopeptidase B-like enzyme, BLM hydrolase, which may be an important determinant of drug action. As seen in Fig. 5, however, the CaM antagonist melittin, pimozide, and chlorpromazine did not inhibit the activity of BLM hydrolase.

Effect of drugs on DNA damage. The cytotoxicity from BLM results from an Fe^{2+}-BLM-O_2 complex that is believed to produce radical species. To test whether CaM antagonists directly affect the ability of BLM to damage DNA, we incubated a covalently closed circular DNA with BLM A_2 in the presence and absence of 10 μM pimozide. Fig. 6 shows the single and double strand DNA breaks caused by BLM A_2. No significant loss of the covalently closed circular pAT 153 DNA (form I) was observed with BLM A_2/DNA molar ratios of 4 or less. With molar ratios of 12 or greater, the single nicked species (form II) was generated under these reaction conditions. Molar ratios of 40 and 100 resulted in the complete loss of form I DNA and the appearance of only form II and form III DNA (linear double nicked DNA). These results are similar to those of Love *et al.* (21). Pimozide had no affect on form I DNA nor did it markedly enhance the appearance of form II and III DNA when added in the presence of BLM A_2.

We next evaluated the actions of a nontoxic concen-

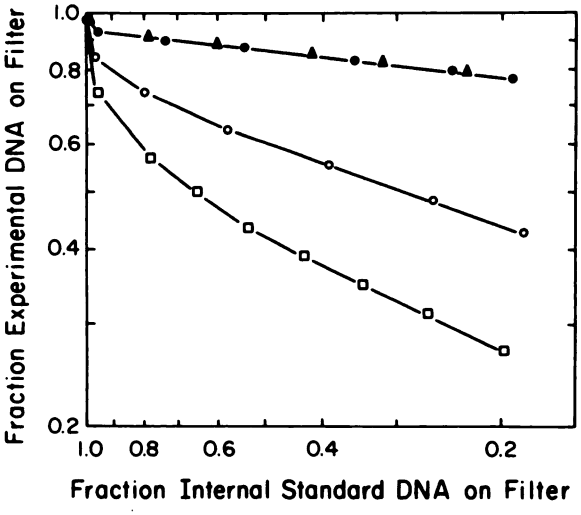

FIG. 7. *DNA strand breakage in intact L1210 cells*

Exponentially growing L1210 cells were incubated with no drug (●), 5 μM pimozide alone (▲), 50 μM BLM A_2 alone (○), or 50 μM BLM A_2 and 5 μM pimozide (□) for 1 hr. The cells were then assayed for DNA breaks by alkaline elution techniques described in Materials and Methods.

TABLE 2

Effects of CaM inhibitors on etoposide and X-irradiation cytotoxicity

Each value is the mean ± standard error of three to seven determinations. Cells were exposed to 1 μM etoposide with or without the CaM inhibitor for 1 hr, washed free of drugs, and assayed for clonal growth as described in Materials and Methods. Cells were X-irradiated with pimozide or vehicle and incubated for 1 hr prior to the removal of pimozide or vehicle and assayed for clonal growth.

	Cell survival		
	Alone	Pimozide (5 μM)	Melittin (2 μM)
	% untreated cells		
Etoposide (1 μM)	18.7 ± 2.4	16.0 ± 2.6	13.8 ± 3.8
X-irradiation (200 rads)	57.9 ± 1.5	61.6 ± 3.1	
X-irradiation (300 rads)	39.0 ± 1.5	33.5 ± 2.7	

tration of pimozide on DNA strand breakage with intact L1210 cells (Fig. 7). Incubation of L1210 cells for 1 hr with 50 μM BLM A_2 caused approximately 170 rad eq of DNA damage as assessed by alkaline elution techniques. The biphasic nature of the alkaline elution profile suggests that small DNA fragments were generated with this concentration of BLM A_2; similar results have been observed by others (3). Lower concentrations produced fewer breaks, e.g., 10 μM BLM A_2 caused approximately 5 rad eq of damage (data not shown). Incubation of L1210 cells simultaneously with 50 μM BLM A_2 and 5 μM pimozide produced a marked increase in DNA strand breaks compared to that seen with BLM A_2 alone. Thus, the potentiation in BLM A_2 cytotoxicity seen with pimozide appears to reflect the enhanced damage to cellular DNA.

Specificity of the potentiation of cytotoxicity. We next examined the effect of CaM antagonists on the cytotoxicity of two treatments that cause repairable DNA strand breakage, the epipodophyllotoxin etoposide (24, 25) and X-irradiation (20). L1210 cells were incubated with 1 μM etoposide in the absence or presence of 5 μM pimozide or 2 μM melittin and survival of cells was measured (Table 2). At a concentration of etoposide that has been reported to cause 300 rad eq of DNA strand breaks in L1210 cells (24), no potentiation of etoposide cytotoxicity was noted with concentrations of pimozide or melittin that greatly enhanced the cytotoxicity of BLM A_2. Similarly, no potentiation of X-irradiation-induced cytotoxicity was seen.

DISCUSSION

The lethal actions of bleomycin are believed to result from DNA strand scissions. Chafouleas *et al.* (13) have observed that the cytotoxicity of bleomycin could be enhanced by a CaM antagonist and proposed that the effect was due to inhibition of repair of DNA damaged by bleomycin. Other possible mechanisms for the enhancement of bleomycin cytotoxicity include (a) an increased accumulation of the drug by altered uptake or steady state levels (26), (b) a reduction in the rate of metabolism of BLM by BLM hydrolase (1), and (c) an augmentation in the ability of bleomycin to produce single or double strand breaks in DNA (1). In our study, we extended the observation of Chafouleas *et al.* (13) by using a variety of structurally dissimilar CaM antagonists and have systematically evaluated their effect on each of these mechanisms. We have employed the principal component of the clinical mixture, BLM A_2, because analytical methods to evaluate its cellular fate and metabolism have now become available (16, 27) and because no evidence exists that the cellular fate or actions of the individual components of the clinical mixture differ.

Agents that increase BLM A_2 uptake have been reported to increase its cytotoxicity (26). Thus, alterations in the rate of BLM A_2 cellular accumulation or steady state levels could offer a potential mechanism for potentiation by CaM antagonists. Our results demonstrate, however, that these agents do not enhance the rate of radioactive BLM A_2 cellular association or increase the steady state levels of cellularly associated BLM A_2 (Fig. 4).

Umezawa (1) and others (16) have found that BLM A_2 is inactivated by a poorly characterized aminopeptidase B-like enzyme, BLM hydrolase. This enzymic activity is believed to be an important factor in determining the toxic properties of BLM to cells. Although L1210 cells contain significant BLM hydrolase activity, our results (Fig. 5) indicate that the activity of BLM hydrolase is unaffected by three different CaM antagonists at concentrations that caused significant enhancement of BLM A_2-induced cell death. Thus, the potentiation with CaM antagonists does not appear to result from altered metabolism of BLM A_2.

The addition of a CaM antagonist to L1210 cells treated with BLM A_2 augmented the number of breaks in DNA, which may be responsible for the increased death of cells. An enhanced breakage of DNA could result from either a direct increase in the destruction of DNA or an indirect effect, such as a decreased repair of DNA damage. Previous studies have demonstrated that extensive repair of DNA damaged by BLM can occur (2, 3). Our results using plasmid DNA (Fig. 6) indicate that in

a cell-free system CaM antagonists do not act by directly increasing the extent of BLM-induced single or double strand breakage of DNA. Rather intact cells are required for CaM antagonists to cause further breakage of DNA (Fig. 7).

CaM antagonists by themselves can exhibit toxicity to cultured cells (8–12). Therefore, it is possible that BLM enhanced the cytotoxicity of these drugs. It seems unlikely, however, that the cytotoxicity of the combination of CaM antagonists and BLM was due solely to an enhancement of the cytotoxicity of CaM antagonists because longer exposure times (8 hr) and higher concentrations (32 μM) of phenothiazines are required to kill L1210 cells (12). In addition, BLM A_2 did not bind to CaM nor did it inhibit the activity of CaM-dependent phosphodiesterase.[2]

Since the original observation by Levin and Weiss (18) that phenothiazines and structurally related drugs bind to and inhibit the activity of CaM, these agents have become useful tools to elucidate the many actions of CaM. These drugs, however, have a variety of pharmacologic activities, some, but not all, being mediated through inhibition of CaM. Consequently, caution is necessary when interpreting results of these and similar studies that use drugs as CaM antagonists. This is especially true since other agents, which have not been reported to inhibit CaM, can enhance BLM cytotoxicity (5, 26). In this study, we have examined a variety of possible mechanisms for the enhancement of BLM-induced cell lethality. These results support the hypothesis that these drugs augment bleomycin cytotoxicity through further damage to DNA, possibly by inhibiting a DNA repair system (13). That the process is regulated by CaM is supported by the observation (a) that the effect was seen with several structurally dissimilar CaM antagonists and (b) that there was a close correlation between the ability of these compounds to antagonize CaM obtained from the target cells and the potentiation of BLM-induced cytotoxicity. To further support the concept that a CaM-mediated DNA repair system exists, Chafouleas *et al.* (13) have shown that the potentiating effect of CaM antagonists on BLM-induced cytotoxicity does not occur in *Escherichia coli*, organisms that lack CaM. Nonetheless, until the CaM-dependent system is identified, other non-CaM-related mechanisms, such as blockade of ion fluxes or membrane perturbations, cannot be completely excluded.

The importance of this hypothesized CaM-mediated mechanism for regulating the cytotoxicity after all DNA damage is unclear. Etoposide and X-irradiation are agents known to cause extensive and repairable single strand breaks in DNA (20, 24, 25), yet we failed to detect significant potentiation by CaM antagonists. These studies indicate that the proposed CaM-mediated process may be important for some but not all cytotoxic agents that cause breakage of DNA.

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[2] W. N. Hait and J. S. Lazo, unpublished observation.

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Send reprint requests to: John S. Lazo, Department of Pharmacology, Yale University School of Medicine, 333 Cedar St., New Haven, CT 06510.